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Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant:

BIONOMICS LIMITED

A.C.N. 075 582 740

Invention Title:

A DIAGNOSTIC METHOD FOR EPILEPSY

The invention is described in the following statement:

A DIAGNOSTIC METHOD FOR EPILEPSY

Background Art

Epilepsies constitute a diverse collection of brain disorders that affect about 3% of the population at some time in their lives (Annegers, 1996). An epileptic seizure can be defined as an episodic change in behaviour caused by the disordered firing of populations of neurons in the central nervous system. This results in varying degrees of involuntary muscle contraction and often a loss of consciousness. Epilepsy syndromes have been classified into more than 40 distinct types based upon characteristic symptoms, types of seizure, cause, age of onset and EEG patterns (Commission on Classification and Terminology of the International League Against Epilepsy, 1989). However the single feature that is common to all syndromes is the persistent increase in neuronal excitability that is both occasionally and unpredictably expressed as a seizure.

A genetic contribution to the aetiology of epilepsy has been estimated to be present in approximately 40% of affected individuals (Gardiner, 2000). As epileptic seizures may be the end-point of a number of molecular aberrations that ultimately disturb neuronal synchrony, the genetic basis for epilepsy is likely to be heterogeneous. There are over 200 Mendelian diseases which include epilepsy as part of the phenotype. In these diseases, seizures are symptomatic of underlying neurological involvement such as disturbances in brain structure or function. In contrast, there are also a number of "pure" epilepsy syndromes in which epilepsy is the sole manifestation in the affected individuals. These are termed idiopathic and account for over 60% of all epilepsy cases.

Idiopathic epilepsies have been further divided into partial and generalized sub-types. Partial (focal or local) epileptic fits arise from localized cortical discharges, so that only certain groups of muscles are

involved and consciousness may be retained (Sutton, 1990). However, in generalized epilepsy, EEG discharge shows no focus such that all subcortical regions of the brain are involved. Although the observation that generalized epilepsies are frequently inherited is understandable, the mechanism by which genetic defects, presumably expressed constitutively in the brain, give rise to partial seizures is less clear.

The idiopathic generalized epilepsies (IGE) are the most common group of inherited human epilepsy and do not have simple inheritance. Two broad groups of IGE are now known - the classical idiopathic generalized epilepsies (Commission on Classification and Terminology of the International League Against Epilepsy, 1989) and the newly recognized genetic syndrome of generalized epilepsy with febrile seizures plus (GEFS$^+$) (Scheffer and Berkovic, 1997; Singh et al., 1999).

The classical IGEs are divided into a number of clinically recognizable but overlapping sub-syndromes including childhood absence epilepsy, juvenile absence epilepsy, juvenile myoclonic epilepsy etc (Commission on Classification and Terminology of the International League Against Epilepsy, 1989; Roger et al., 1992). The sub-syndromes are identified by age of onset and the pattern of seizure types (absence, myoclonus and tonic-clonic). Some patients, particularly those with tonic-clonic seizures alone do not fit a specifically recognized sub-syndrome. Arguments for regarding these as separate syndromes, yet recognizing that they are part of a neurobiological continuum, have been presented previously (Berkovic et al. 1987; 1994; Reutens and Berkovic, 1995).

GEFS$^+$ was originally recognized through large multi-generation families and comprises a variety of sub-syndromes. Febrile seizures plus (FS$^+$) is a sub-syndrome where children have febrile seizures occurring outside the age range of 3 months to 6 years, or have associated febrile tonic-clonic seizures. Many family members have a

phenotype indistinguishable from the classical febrile convulsion syndrome and some have FS$^+$ with additional absence, myoclonic, atonic, or complex partial seizures. The severe end of the GEFS$^+$ spectrum includes myoclonic-astatic epilepsy.

In GEFS$^+$ families, linkage analysis on rare multi-generation large families with clinical evidence of a major autosomal dominant gene have demonstrated loci on chromosomes 19q and 2q. Both the 19q and 2q GEFS$^+$ loci have been confirmed in independently ascertained large families, and genetic defects have been identified. Families linked to 19q are known and a mutation in the gene for the β1 subunit of the neuronal sodium channel (SCN1B) has been identified (Wallace et al., 1998). This mutation results in the loss of a critical disulphide bridge of this regulatory subunit and causes a loss of function *in vitro*. Families linked to 2q are also known and mutations in the pore-forming α subunit of the neuronal sodium channel (SCN1A) have been identified (PCT/AU01/01648; Escayg et al., 2000).

Severe myoclonic epilepsy of infancy (SMEI) is classed as an epileptic syndrome that manifests as both generalised and focal (partial) seizures (Commission on Classification and Terminology of the International League Against Epilepsy, 1989). SMEI begins with prolonged febrile and afebrile hemiclonic and generalised seizures in the first year of life. Between one and four years, other seizure types evolve including myoclonic, absence and atonic seizures. Neurological development is normal in infancy with progressive slowing after two years. A family history of epilepsy and/or febrile seizures is often found in SMEI patients and recent work has shown that family members have epilepsy phenotypes consistent with the GEFS+ spectrum (Singh et al., 2001; Veggiotti, 2001). From a clinical perspective, as GEFS+ and SMEI involve fever-related seizures, it was thought that sodium channel genes may be the target for mutations in SMEI affected

individuals. This fact was later confirmed when mutations in the SCN1A gene in SMEI patients were identified (Claes et al., 2001; Ohmori et al., 2002). Of interest is that each of these mutations were *do novo*, a fact difficult to reconcile based on the clinical experience that a significant number of SMEI cases have a family history of GEFS+.

The inventors have established that SCN1A is the most frequently mutated gene causative for SMEI based on studies to date (Mulley et al., 2003). Based on this fact the inventors have established a method for the diagnosis of SMEI based on the identification of mutations (novel and known) in the SCN1A gene.

Disclosure of the Invention

In a first aspect of the present invention there is provided a method of identifying SMEI-associated mutations in the SCN1A gene comprising the steps of selecting a system of assays comprising at least a first and final assay, said first assay being selected to provide a test for the existence of a SMEI-associated mutation and said final assay being selected to provide a test to determine the nature of the SMEI-associated mutation. There are a number of assay systems that may be selected to perform the diagnostic test and these are known to one skilled in the art. Examples are provided below.

In a second aspect of the present invention there is provided a method of testing patients for SMEI-associated mutations in the SCN1A gene comprising the steps of:

(1) selecting a system of assays comprising at least a first and final assay, said first assay being selected to provide a test for the existence of a SMEI-associated mutation and said final assay being selected to provide a test to determine the nature of the SMEI-associated mutation;

(2) performing said first assay; and, if the results indicate the existence of a SMEI-associated mutation,

(3) performing said second assay.

In one embodiment an assay system employed may be the analysis of SCN1A DNA from a patient sample in comparison to wild-type SCN1A DNA. Genomic DNA may be used for the diagnostic analysis and may be obtained from body cells, such as those present in the blood or cheek, tissue biopsy, surgical specimen, or autopsy material. The DNA may be isolated and used directly for the diagnostic assays or may be amplified by the polymerase chain reaction (PCR) prior to analysis. Similarly, RNA or cDNA may also be used, with or without PCR amplification.

In a specific embodiment, a DNA hybridisation assay may be employed. These may consist of probe-based assays specific for the SCN1A gene. In an SCN1A exon specific assay, the probe-based assay will utilize at least one probe which specifically and selectively hybridises to an exon of the SCN1A gene in its wild-type form. Thus, the lack of formation of a duplex nucleic acid hybrid containing the nucleic acid probe is indicative of the presence of the mutation in the SCN1A gene. Because of the high specificity of probe-based tests, any negative result is highly indicative of the presence of the SMEI-associated mutation and further investigational assays should be employed to determine the nature of the mutation.

The SCN1A exon specific assay approach could also be adapted to identify previously determined SCN1A mutations responsible for SMEI. In this aspect, a probe which specifically and selectively hybridises with the SCN1A gene in its mutant form is used. In this case the formation of a duplex nucleic acid hybrid containing the nucleic acid probe is indicative of the presence of the mutation in the SCN1A gene. In each variation of the exon specific assay approach, it is important to take into

account known polymorphisms in the SCN1A gene that are not associated with SMEI. A secondary assay such as DNA sequencing should subsequently be employed to ensure that any suspected mutations are not known polymorphisms.

The exon specific probes used for these assays may be derived from: (1) PCR amplification of each exon of the SCN1A gene using intron specific primers flanking each exon; (2) cDNA probes specific for each exon; or (3) a series of oligonucleotides that collectively represent an SCN1A exon.

In a further embodiment, patient samples may be subject to electrophoretic-based assays. For example electrophoretic assays that determine SCN1A fragment length differences may be employed. Fragments of each patient's genomic DNA are amplified with SCN1A gene intron specific primers. The amplified regions of the SCN1A gene therefore include the exon of interest, the splice site junction at the exon/intron boundaries, and a short portion of intron at either end of the amplification product. The amplification products may be run on an electrophoresis size-separation gel and the lengths of the amplified fragments are compared to known and expected standard lengths from the wild-type gene to determine if an insertion or deletion mutation is found in the patient sample. An SCN1A gene mutation may be diagnosed if such length variations are identified. This procedure can advantageously be used in a "multiplexed" format, in which primers for a plurality of exons (generally from 2 to 8) are co-amplified, and evaluated simultaneously on a single electrophoretic gel. This is made possible by careful selection of the primers for each exon. The amplified fragments spanning each exon are designed to be of different sizes and therefore distinguishable on an electrophoresis/size separation gel. The use of this technique has the advantage of detecting both normal and mutant alleles in heterozygous individuals. Furthermore,

through the use of multiplexing it can be very cost effective.

In a further approach, diagnostic electrophoretic assays for the detection of previously identified SCN1A mutations may utilise PCR primers which bind specifically to mutated exons of the SCN1A gene. In this case, product will only be observed in the electrophoresis gel if hybridization of the primer occurred. Thus, the appearance of amplification product is an indicator of the presence of the mutation, while the length of the amplification product may indicate the presence of additional mutations.

Additional electrophoretic assays may be employed. These may include the single-stranded conformational polymorphism (SSCP) procedure. As mentioned above, fragments of each patient's genomic DNA are PCR amplified with SCN1A gene intron specific primers such that individual exons of the SCN1A gene are amplified and may be analysed individually. Exon-specific PCR products are then subjected to electrophoresis on non-denaturing polyacrylamide gels such that DNA fragments migrate through the gel based on their conformation as dictated by their sequence composition. SCN1A exon-specific fragments that vary in sequence from wild-type SCN1A sequence will have a different secondary structure conformation and therefore migrate differently through the gel. Aberrantly migrating PCR products in patient samples are indicative of a mutation in the SCN1A exon and should be analysed further in secondary assays such as DNA sequencing to determine the nature of the mutation.

In a further embodiment, enzymatic based assays may be used in diagnostic applications. Such assays include the use of S1 nuclease, ribonuclease, T4 endonuclease VII, MutS, Cleavase and MutY.

When a diagnostic assay is to be based upon the SCN1A protein, a variety of approaches are possible. For example, diagnosis can be achieved by monitoring differences in the electrophoretic mobility of normal

SCN1A protein and SCN1A protein isolated from a test sample. Such an approach will be particularly useful in identifying mutants in which charge substitutions are present, or in which insertions, deletions or substitutions have resulted in a significant change in the electrophoretic migration of the resultant protein. Alternatively, diagnosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the various amino acid residues, or by functional assays demonstrating altered function of the gene products.

Further assays that are based on the SCN1A protein include immunoassays. Immunoassays for the SCN1A gene product are not currently known. However, immunoassay is included in the selection of assays because the procedures for raising antibodies against specific gene products are well described in the literature, for example in U.S. Pat. Nos. 4,172,124 and 4,474,893 which are incorporated herein by reference. Antibodies are normally raised which bind to portions of the gene product away from common mutation sites such that the same antibody binds to both mutant and normal protein. Preferred antibodies for use in this invention are monoclonal antibodies because of their improved predictability and specificity. It will be appreciated, however, that essentially any antibody which possesses the desired high level of specificity can be used, and that optimization to achieve high sensitivity is not required.

For the diagnostic detection of novel mutations in SCN1A involved in SMEI, antibodies raised to the carboxy-terminal end of the protein would be preferable. For the diagnostic detection of mutations previously identified in SCN1A, antibody raised against the defective gene product is preferable. Antibodies are added to a portion of the patient sample under conditions where an immunological reaction can occur, and the sample is then evaluated to see if such a reaction has occurred. The specific method

for carrying out this evaluation is not critical and may include enzyme-linked immunosorbant assays (ELISA), described in U.S. Pat. No. 4,016,043, which is incorporated herein by reference; fluorescent enzyme immunoassay (FEIA or ELFA), which is similar to ELISA, except that a fluoregenic enzyme substrate such as 4-methylumbelliferyl-beta-galactoside is used instead of a chromogenic substrate, and radioinmunoassay (RIA).

The most definitive diagnostic assay that may be employed is DNA sequencing. Comparison of the SCN1A DNA wild-type sequence with the SCN1A sequence of a test patient provides both high specificity and high sensitivity. The general methodology employed involves amplifying (for example with PCR) the DNA fragments of interest from patient DNA; combining the amplified DNA with a sequencing primer which may be the same as or different from the amplification primers; extending the sequencing primer in the presence of normal nucleotide (A, C, G, and T) and a chain-terminating nucleotide, such as a dideoxynucleotide, which prevents further extension of the primer once incorporated; and analyzing the product for the length of the extended fragments obtained. While such methods, which are based on the original dideoxysequencing method disclosed by Sanger et al., 1977 are useful in the present invention, the final assay is not limited to such methods. For example, other methods for determining the sequence of the gene of interest, or a portion thereof, may also be employed. Alternative methods include those described by Maxam and Gilbert (1977) and variations of the dideoxy method and methods which do not rely on chain-terminating nucleotides at all such as that disclosed in U.S. Pat. No. 4,971,903, which is incorporated herein by reference. Any sequence differences (other than benign polymorphisms) in SCN1A exons of a test patient when compared to that of the wild-type SCN1A sequence indicate a potential disease-causing mutation.

In specific embodiments of the invention, there is provided a method for testing patients for SMEI-associated mutations in the SCN1A gene comprising the steps of:

a) quantitatively amplifying at least one exon of the SCN1A gene from a body sample of each patient to produce amplified fragments;

b) comparing the properties of the amplified fragments to standard values based upon the fragments produced by amplification of the same exon in a non-mutant SCN1A gene; and

c) determining the nucleic acid sequence of each exon identified in b) that has different properties in the patient compared to the corresponding non-mutant SCN1A exon.

In further specific embodiments there is provided a method for testing patients for SMEI-associated mutations in the SCN1A gene comprising the steps of:

a) quantitatively amplifying, from a body sample of each patient at least one exon of the SCN1A gene using primers complementary to intron regions flanking each amplified exon;

b) comparing the length of the amplification products for each amplified exon to the length of the amplification products obtained when a wild-type SCN1A gene is amplified using the same primers, whereby differences in length between an amplified sample exon and the corresponding amplified wild-type exon reflect the occurrence of a truncating mutation in the sample SCN1A gene; and

c) determining the nucleic acid sequence of each exon identified in b) to contain a truncating mutation.

In even further specific embodiments there is provided a method for testing patients for SMEI-associated mutations in the SCN1A gene comprising the steps of:

a) quantitatively amplifying, from a body sample of each patient at least one exon of the SCN1A gene using

primers complementary to intron regions flanking each amplified exon;

b) hybridising the fragments from a) with fragments produced by amplification of the same exon in a non-mutant SCN1A gene;

c) determining the nucleic acid sequence of each patient exon identified in b) that either does not hybridise to corresponding wild-type fragments or forms a mismatched heteroduplex as detected by an enzymatic assay.

Throughout this specification and the claims, the words "comprise", "comprises" and "comprising" are used in a non-exclusive sense, except where the context requires otherwise.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

Modes for Performing the Invention

Any combination of assay systems described above may be employed for the diagnosis of SCN1A mutations responsible for SMEI. Provided below is an example of an assay combination.

Example 1: SCN1A electrophoretic assay using SSCP

DNA from a test patient may be obtained in a number of ways. The most common approach is to obtain DNA from blood samples taken from the patient, however DNA may also be obtained using less invasive approaches such as from cheek cell swabs.

For this specific example DNA was extracted from collected blood using the QIAamp DNA Blood Maxi kit (Qiagen) according to manufacturers specifications or

through procedures adapted from Wyman and White (1980). Stock DNA samples were kept at a concentration of 1 ug/ul.

Once DNA is obtained, PCR amplification of individual exons of the SCN1A gene may be employed prior to analysis by SSCP. Table 1 provides a list of primers that may be employed to analyse each exon of the SCN1A gene.

In this specific example, primers used for SSCP were labelled at their 5′ end with HEX and typical PCR reactions were performed in a total volume of 10 µl. All PCR reactions contained 67 mM Tris-HCl (pH 8.8); 16.5 mM $(NH_4)_2SO_4$; 6.5 µM EDTA; 1.5 mM $MgCl_2$; 200 µM each dNTP; 10% DMSO; 0.17 mg/ml BSA; 10 mM β-mercaptoethanol; 5 µg/ml each primer and 100 U/ml *Taq* DNA polymerase. PCR reactions were performed using 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds followed by 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. A final extension reaction for 10 minutes at 72°C followed.

Twenty µl of loading dye comprising 50% (v/v) formamide, 12.5 mM EDTA and 0.02% (w/v) bromophenol blue were added to completed reactions which were subsequently run on non-denaturing 4% polyacrylamide gels with a cross-linking ratio of 35:1 (acrylamide:bis-acrylamide) and containing 2% glycerol. Gel thickness was 100µm, width 168mm and length 160mm. Gels were run at 1200 volts and approximately 20mA, at 22°C and analysed on the GelScan 2000 system (Corbett Research, Australia) according to manufacturers specifications.

Example 2: DNA sequencing assay

PCR products from the SSCP analysis that showed a conformational change may be subject to secondary assays such as DNA sequencing to determine the nature of the change. In the example provided here, this first involved re-amplification of the amplicon displaying a band-shift from the relevant individual (primers used in this instance did not contain 5′ HEX labels) followed by

purification of the PCR amplified templates for sequencing using QiaQuick PCR preps (Qiagen) based on manufacturers procedures. The primers used to sequence the purified amplicons were identical to those used for the initial amplification step. For each sequencing reaction, 25 ng of primer and 100 ng of purified PCR template were used. The BigDye sequencing kit (ABI) was used for all sequencing reactions according to the manufacturers specifications. The products were run on an ABI 377 Sequencer and analysed using the EditView program.

A comparison of the DNA sequence obtained from the patient sample can then be made directly to that of the wild-type SCN1A sequence in order to detect the DNA alteration that lead to the conformational change detected by SSCP. If the DNA change is not a known polymorphism in the SCN1A gene, it is likely that it may be a disease causing mutation essentially providing a diagnosis that can be investigated further through the analysis of additional family members.

TABLE 1

Primer Sequences Used for Mutation Analysis of SCN1A

Exon	Forward Primer	Reverse Primer	Size(bp)
1A	TACCATAGAGTGAGGCGAGG	ATGGACTTCCTGCTCTGCCC	356
1	CCTCTAGCTCATGTTTCATGAC	TGCAGTAGGCAATTAGCAGC	448
2	CTAATTAAGAAGAGATCCAGTGACAG	GCTATAAAGTGCTTACAGATCATGTAC	356
3	CCCTGAATTTTGGCTAAGCTGCAG	CTACATTAAGACACAGTTTCAAAATCC	263
4	GGGCTACGTTTCATTTGTATG	GCAACCTATTCTTAAAGCATAAGACTG	355
5	AGGCTCTTTGTACCTACAGC	CATGTAGGGTCCGTCTCATT	199
6	CACACGTGTTAAGTCTTCATAGT	AGCCCCTCAAGTATTTATCCT	394
7	GAACCTGACCTTCCTGTTCTC	GTTGGCTGTTATCTTCAGTTTC	241
8	GACTAGGCAATATCATAGCATAG	CTTTCTACTATATTATCATCCGG	320
9	TTGAAAGTTGAAGCCACCAC	CCACCTGCTCTTAGGTACTC	363
10	GCCATGCAAATACTTCAGCCC	CACAACAGTGGTTGATTCAGTTG	480
11a	TGAATGCTGAAATCTCCTTCTAC	CTCAGGTTGCTGTTGCGTCTC	306
11b	GATAACGAGAGCCGTAGAGAT	TCTGTAGAAACACTGGCTGG	315
12	CATGAAATTCACTGTGTCACC	CAGCTCTTGAATTAGACTGTC	347
13a	ATCCTTGGGAGGTTTAGAGT	CATCACAACCAGGTTGACAAC	292
13b	CTGGGACTGTTCTCCATATTG	GCATGAAGGATGGTTGAAAG	277
14	CATTGTGGGAAAATAGCATAAGC	GCTATGCAGAACCCTGATTG	338
15a	TGAGACGGTTAGGGCAGATC	AGAAGTCATTCATGTGCCAGC	348
15b	CTGCAAGATCGCCAGTGATTG	ACATGTGCACAATGTGCAGG	276
16a	GTGGTGTTTCCTTCTCATCAAG	TCTGCTGTATGATTGGACATAC	387
16b	CAACAGTCCTTCATTAGGAAAC	ACCTTCCCACACCTATAGAATC	353
17	CTTGGCAGGCAACTTATTACC	CAAGCTGCACTCCAAATGAAAG	232
18	TGGAAGCAGAGACACTTTATCTAC	GTGCTGTATCACCTTTTCTTAATC	234
19	CCTATTCCAATGAAATGTCATATG	CAAGCTACCTTGAACAGAGAC	318
20	CTACACATTGAATGATGATTCTGT	GCTATATACAATACTTCAGGTTCT	216
21a	ACCAGAGATTACTAGGGGAAT	CCATCGAGCAGTCTCATTTCT	303
21b	ACAACTGGTGACAGGTTTGAC	CTGGGCTCATAAACTTGTACTAAC	297
22	ACTGTCTTGGTCCAAAATCTG	TTCGATTAATTTTACCACCTGATC	267
23	AGCACCAGTGACATTTCCAAC	GGCAGAGAAAACACTCCAAGG	272
24	GACACAGTTTTAACCAGTTTG	TGTGAGACAAGCATGCAAGTT	207
25	CAGGGCCAATGACTACTTTGC	CTGATTGCTGGGATGATCTTGAATC	477
26a	CGCATGATTTCTTCACTGGTTGG	GCGTAGATGAACATGACTAGG	247
26b	TCCTGCGTTGTTTAACATCGG	ATTCCAACAGATGGGTTCCCA	288
26c	TGGAAGCTCAGTTAAGGGAGA	AGCGCAGCTGCAAACTGAGAT	261
26d	CCGATGCAACTCAGTTCATGGA	GTAGTGATTGGCTGATAGGAG	274
26e	AGAGCGATTCATGGCTTCCAATCC	TGCCTTCTTGCTCATGTTTTTCCACA	335
26f	CCTATGACCGGGTGACAAAGCC	TGCTGACAAGGGGTCACTGTCT	242

Note: Primer sequences are listed 5' to 3'. Due to the large size of exons 11, 13, 15, 16, 21 and 26, the exons were split into two or more overlapping amplicons.

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